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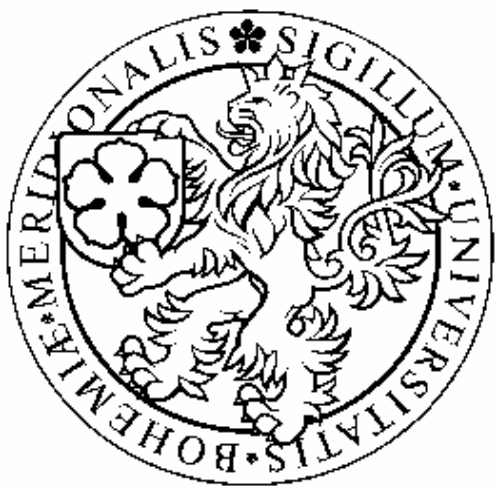

IrAM4: Partial characterisation of a molecule similar to α_2-macroglobulin from a tick *Ixodes ricinus*

Bachelor Thesis

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Annotation

Ixodes ricinus is a hard tick that can transmit several diseases that are capable of affecting humans. Among those are tick-borne encephalitis and Lyme disease. The focus of this work is on IrAM4, a member of tick α_2-macroglobulin family (α_2M-F) of proteins which belong to the evolutionarily oldest constituents of the innate immune system. α_2-macroglobulins are protease inhibitors and act primarily in inactivation of proteases secreted by invading pathogens within their infection cycle. The aim of this study was to identify α_2-M of *Ixodes ricinus* (IrAM4) from corresponding ortholog of protein α_2-M named IsAM4 present in the genome of closely related *Ixodes scapularis.* The partial sequence was determined by amplification of cDNA and subsequent sequencing of PCR products. RT-PCR tissue profiling revealed that IrAM4 is present in ovaries and salivary glands but not in the tick gut. The recombinant fragment of IrAM4 was afterwards prepared for immunization of a rabbit and the obtained polyclonal antibodies were used for Western blot analysis. The results showed that IrAM4 is mainly present in the hemolymph and probably in salivary glands and ovaries but it is not expressed in the gut. The native IrAM4 seems to be composed of two disulfide bound subunits. However, the exact structure of the molecule was not analyzed in this work.

Affirmation

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České Budějovice, May 20, 2011 Markéta Absolonová

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1. INTRODUCTION

Ticks are among the most important vectors of disease to humans throughout the world. Among the diverse microbes transmitted by ticks are several protozoan, viral, and bacterial pathogens. Hard ticks, called Ixodidae, cause most tick-borne illness in humans. The name hard ticks originates from the presence of a hard plate (sputum), covering the dorsal body surface. Since humans are atypical hosts, tick borne illness is usually acute and at times severe, disabling, or even fatal. Tick bites can cause severe allergic reactions, irritations, various types of tick toxicoses, and tick paralysis [1,2].

The hard tick's life cycle includes the three parasitic stages of larva, nymph and adult. The engorged larva drops off, molts to a nymph, which then has to find a second host animal on which it engorges and drops off again to molt to the adult stage, which attaches to a third host animal [3].

Ticks, being obligate blood feeding ectoparasites, of course exhibit a major dependence on the ability to digest host proteins. According to this one imaginable strategy on the way to retarding disease transmission could be the disruption of this digestion ability. However, the constituent proteinase in the parasite gut and their potential interaction in the digestion of the blood haven't been clearly understood yet. Digestion of host proteins, including hemoglobin, by gut-associated proteinase and peptidase is the most important activity in providing nutrition and energy for parasite molting and vitellogenesis [4].

Proteases play an important role in parasitic metabolism and virulence. They function in invasion, immune evasion, nutrition and reproduction. The immune system of the host acts against invading parasites by producing inhibitors that are capable of inactivating the proteases and thus are an important component of the immune system of parasites. Protease inhibitors can be distinguished into two functional classes, active-site inhibitors, which bind to the active sites of proteases and inactivate them, and the α_2-macroglobulins (α_2-M) [5].

α_2-M are protease inhibitors with a wide range of specifity [6]. The main features of this family are a broad reactive capacity against proteases of all classes, a unique mechanism of molecular entrapment of the target proteases and inability to inactivate the active site of the entrapped protease. Another feature of the members of the α_2-macroglobulin family (α_2M-F) of proteins is the presence of a reactive internal thiol ester bond. The thiol ester proteins also share similar sequences and similar domain structure [7,8].

Proteins of the α_2-macroglobulin family are abundant components of arthropod and mammalian plasma [9,10]. The proteins of α_2M-F in invertebrates are divided into four subfamilies: (a) α_2-macroglobulins (α_2-M), (b) complement factors C3/C4/C5 (c) invertebrate insect thioester-containing proteins (TEPs), which have been identified in the fruitfly *Drosophila melangoster* [11] and in a mosquito vector of human malaria, *Anopheles gambiae* [12], and (d) macroglobulin-complement related molecules (MCR-like) whereas the function of the latter remains unknown in the case of insect species. [8,13].

α_2-M has been characterized and purified in invertebrates from gastropod mollusc *Biomhalaria glabrata* [14] as well as from arthropods such as American horseshoe crab *Limulus polyphemus* [15], mud crab *Scylla serrata* [16], shrimps *Penaues vannamei* [17], *Penaeus monodon* [18] and *Marsupenaeus japonicus* [19] and from two ticks *Ornithodoros moubata* [20] and *Ixodes ricinus* [21].

The *Ixodes scapularis* genome database contain nine proteins of α_2M-F named as IsAM 1-9 *(Figure 1)*. The corresponding homologues in *Ixodes ricinus* have been identified and proven to be almost identical to *Ixodes scapularis* and thus can allow parallel functional genomic studies in both tick species. IrAM1, IrAM2 and IrAM4 are clustered to α_2-M protease inhibitors, IrAM3 to TEPs and IrAM5, IrAM6 and IrAM7 belong to complement components. IrAM8 and IrAM9 form a fourth group named macroglobulin related molecules [22].

The aim of this work was to characterize the fragment of α_2-macroglobulin IrAM4 from the hard tick *Ixodes ricinus*. The corresponding ortholog could be identified by the help of primers derived from the genome *of Ixodes scapularis* due to high similarity of those two ticks. The expression of IrAM4 in various tissues was investigated by RT-PCR method. A recombinant protein has been prepared in order to obtain polyclonal serum by repeated immunization of a rabbit. The polyclonal serum was further used for Western blot analysis.

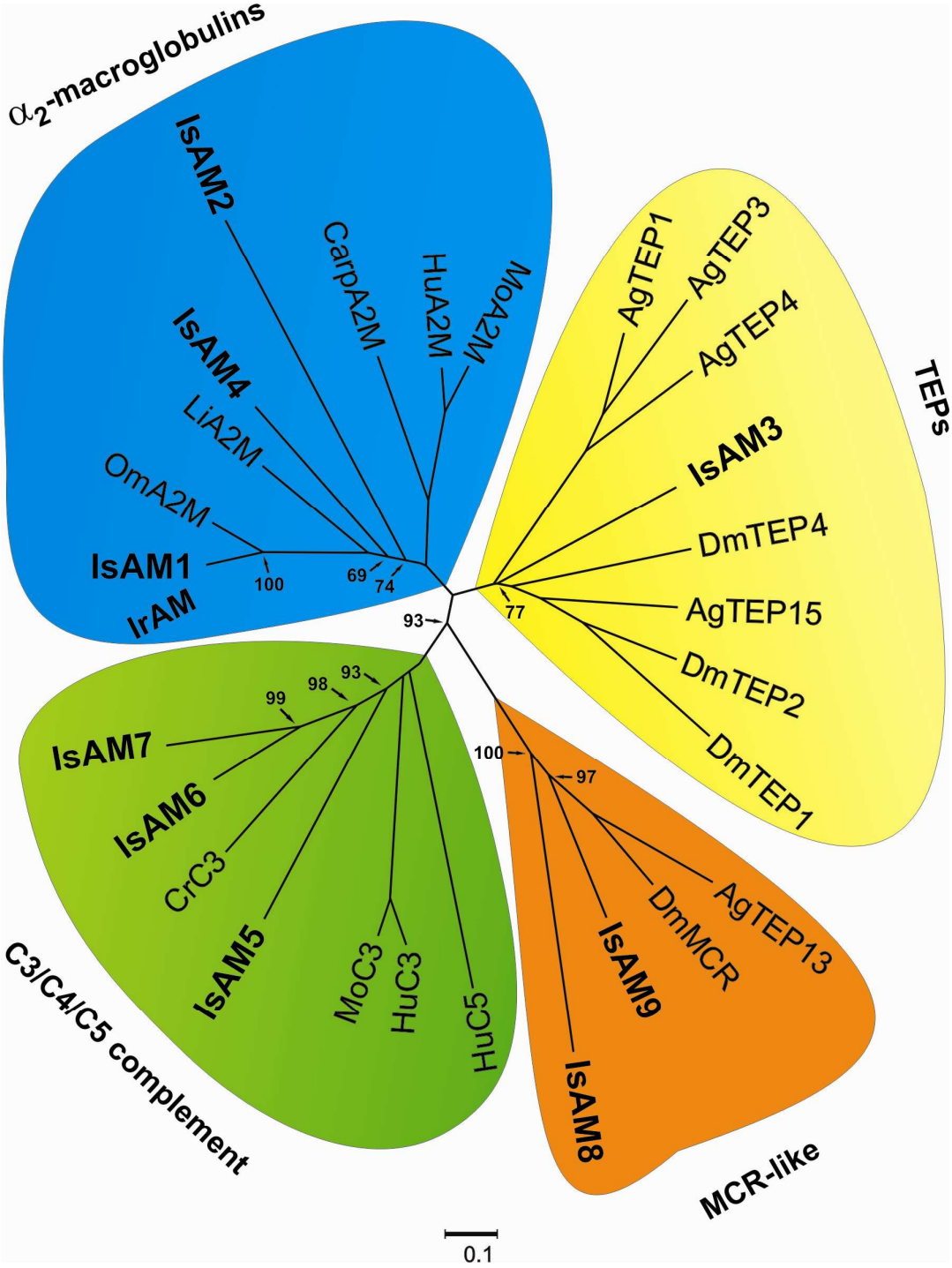

Figure 1: Phylogenetic tree of proteins of α_2-macroglobulin family
The scheme of the phylogentic tree was adapted from a study by Burešová et al. [22].
α_2M-macroglobulin protease inhibitor group is in blue. HuA2M (Homo sapiens), *MoA2M* (Mus musculus), *CarpA2M* (Cyprinus carpio), *LiA2M* (Limulus sp.), *OmA2M* (Ornithodoros moubata). *The group of complement components is in green and the sub-group of Mcr-like proteins is in orange. CrC3* (Carcinoscorpius rotundicauda), *HuC3* (Homo sapiens), *MoC3* (Mus musculus), *AgTEP13* (Anopheles gambiae), *DmMcr* (Drosophila melanogaster). *TEPs family is displayed in yellow. AgTEP1, AgTEP3* and *AgTEP4* (Anopheles gambiae), *DmTEP1, DmTEP2 and DmTEP4* (Drosophila melanogaster). *IsAM1, IsAM2, IsAM3, IsAM4, IsAM5, IsAM6, IsAM8 and IsAM9* (Ixodes scapularis).

2. MATERIALS AND METHODS

2.1. Collection of ticks

Adult females of *Ixodes ricinus* were collected in České Budějovice in the Czech Republic. Adult females of *Ixodes ricinus* were fed on laboratory guinea pigs for five days.

2.2. Tissue dissections and hemolymph collection

Hemolymph, obtained by cutting off the ticks legs, was collected into a glass capillary. Tissues were dissected on a Petri dish filled with wax and rinsed in PBS buffer (phosphate buffered saline). Tissues used for the RNA isolation were placed into vials with TRI Reagent ™ solution (Invitrogen). Tissues used for the immunodetection were placed into vials with 1 x DTT buffer (dithiothreitol).

2.3. Total RNA isolation

The tissues (salivary glands, gut and ovaries) were placed into vials with the TRI Reagent ™ solution (Invitrogen), 1 mL/100 mg of freshly dissected tissue, and homogenized with a plastic pestle. Chloroform was added to each vial, 200 µL of chloroform/1 000 µL of TRI Reagent™. The homogenate was then centrifuged (4 °C, 12 000 rpm, 15 min) and the supernatant containing RNA was placed into a vial, where the isolation was performed according to enclosed directions of TRI Reagent™. The quality of total RNA was checked by electrophoresis on a 1.2 % TBE agarose gel and the concentration of total RNA was measured on a spectrometer at λ = 260 nm.

2.4. Synthesis of cDNA

Total RNA isolated from the tissues was diluted to the final concentration of 0.5 µg/µL and transcribed into cDNA by using anchored-oligo $(dT)_{18}$ primer and Transcriptor High Fidelity cDNA Synthesis Kit (Roche™) following the provided protocol.

2.5. Amplification of DNA

The amplification was performed by using gene-specific primers from *Ixodes scapularis* designed for *Ixodes ricinus (Table 1)* [13]. Specific DNA fragments were amplified by using PCR (35 cycles, annealing temperature = 55°C) using Taq DNA polymerase (Invitrogen) and the

template cDNA from the salivary glands of *Ixodes ricinus*. The amplification conditions for primers were experimentally determined by testing an optimal annealing temperature using thermal cycler MasterGradient (Eppendorf). PCR products were isolated from 1 % agarose gel by using QIAquick Gel Extraction Kit (250) (QIAGEN).

Table 1: Primers used for the amplification of IrAM4 fragment

Primer	Sequence
IrAM4-RT S	5'- GGT CTC TGG ATC TTC AAG GG -3'
IrAM4-RT AS	5'- GGA CGT ATG CTG TCA GTG G -3

2.6. Transcriptional profiling by RT-PCR

For RT-PCR method, cDNA from tick tissues was diluted 10 times in DEPC water and 1 µL of the template was used in 10 µL of PCR reaction solution. The PCR product was amplified (35 cycles, at annealing temperature t = 58 °C). Ferritin was applied as a loading control.

2.7. DNA cloning into vector and sequencing

Obtained PCR products were cloned into the pCR 2.1®-TOPO® vector using the TOPO TA Cloning Kit® (Invitrogen) and transformed by heat shock method (42 °C, 45 s) into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen). Blue/white screening was used for selection of desired colonies. Selected colonies of *E. coli* were cultivated in LB medium containing ampicillin (50 µg/ml). Plasmid DNA was isolated by using High Pure Plasmid Isolation Kit (Roche). The proper cloning was verified by restriction of plasmids by EcoRI FastDigest® (Fermentas) restriction enzyme and DNA was sequenced in both directions by an automated sequencer (Applied Biosystems 3130x1 (Hitachi), at Laboratory of genomics, Academy of Sciences in the Czech Republic, České Budějovice) using M13 S and M13 AS primers *(Table 2)*. The sequences were edited and assembled using programs DNA STAR and CHROMAS.

Table 2: Primers for the DNA sequencing

Primer	Sequence
M13 S	5'- GTA AAA CGA CGG CCA -3'
M13 AS	5'- CAG GAA ACA GCT ATG AC -3'

2.8. Recombinant protein expression

The bacterial system of *E. coli* Champion™ Directional Expression Kit (Invitrogen) was applied for the expression of the recombinant protein. Histidine-tagged fusion protein was prepared using pET100/D-TOPO® (Invitrogen). The insert was prepared using PCR product with specific IrAM4pET100 S and IrAM4pET100 AS primers *(Table 3)* and cDNA from salivary glands of *Ixodes ricinus* was used as a template. The insert was subsequently transformed into TOP10 Chemically Competent *E. coli* (Invitrogen) and the sequence and correct ORF of the subcloned product were verified by the automated sequencer (Applied Biosystems 3130x1 (Hitachi)) using T7 S and T7 AS primers *(Table 3)*. The verified construct was afterwards transformed into One Shot® BL21 Star™ (DE3) Chemically Competent *E. coli* (Invitrogen). The bacterial culture of plasmid containing BL21 competent cells was mixed with LB medium containing ampicillin (50 µg/mL) and incubated over night (37 °C, 200 rpm). Overnight culture was inoculated into fresh LB medium (10 mL with 200 mL of LB media) and IPTG solution (isopropylthio-β-D-galactoside) was added to 0.5 mM final concentration when OD_{600} = 0.6 and the culture incubated for another 6 hours at 37 °C and 200 rpm (verified by pilot expression). After 6 hours the bacterial culture was centrifuged (4 °C, 6 000 rpm, 20 min). Collected cells were frozen and stored at -20 °C. The pilot expression was used to optimize the expression levels. 0.5 mL of the bacterial culture was taken away every 1.5 hours and shortly centrifuged (4 °C, 6 000 rpm, 1 min). The cells were lysed using homogenization buffer *(Table 4)* and repeated freezing and thawing in liquid nitrogen. 1 x DTT was added to each sample and the rate of expression was checked by SDS-PAGE on a gradient polyacrylamide gel (4.5 - 17 %).

Table 3: Primers used for the recombinant protein expression

Primer	Sequence
IrAM4pET100 S	5'- CAC CAG GGG TGT TTT TAC GCT TTC AGG -3'
IrAM4pET100 AS	5'- TTA ACA TCG CAC GGC ACC CAC TCT C -3'
T7 S	5´- TAA TAC GAC TCA CTA TAG GG -3´
T7 AS	5´- GCT AGT TAT TGC TCA GCG G -3´

Table 4: Composition of the homogenization buffer

Homogenization buffer	150 mM KCl 10 mM Tris 1 mM EDTA (Ethylenediaminetetraacetic acid) 1 mM PMSF (Phenylmethylsulfonyl fluoride) 5 mM mercaptoethanol

2.9. Isolation of inclusion bodies

The bacterial pellet was dissolved in a resuspension buffer *(Table 5)*, sonicated 4x10 s on ice and centrifuged (4 °C, 13 000 rpm, 10 min). Then the pellet was dissolved in an isolation buffer and again sonicated 4x10 s on ice and centrifuged (4 °C, 13 000 rpm, 10 min). The procedure with the resuspension buffer was repeated once. Then the pellet was dissolved in a solubilization buffer and centrifuged at room temperature (13 000 rpm, 30 min). Supernatant was filtrated through 0.22 µm Millex® GP (Millipore).

Table 5: Composition of buffers for the isolation of inclusion bodies

Resuspension buffer	20 mM Tris/HCl pH = 8
Isolation buffer	20 mM Tris/HCl 2 M urea 0.5 M NaCl 10 mM imidazol pH = 8
Solubilization buffer	20 mM Tris/HCl 1 mM mercaptoethanol 10 mM imidazol 0.5 M NaCl 6 M guanidine hydrochloride pH = 8

2.10. Recombinant His-tagged protein purification

The Histidine-tagged fusion protein was purified from the solubilized inclusion bodies by Co^{2+} chelate column using the AKTA™FPLC™ chromatographic system (GE Healthcare). The purification buffers are noted in *Table 6*. The recombinant protein was eluted with linear gradient of imidazol, fractions were collected and the purity of the fusion protein preparation in eluted fractions was visually assessed using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) on a gradient polyacrylamide gel (4 – 17.5 %).

Table 6: Composition of purification buffers

Buffer A	8 M urea 50 mM Tris/HCl 0.5 M NaCl pH = 7.8
Buffer B	8 M urea 50 mM Tris/HCl 0.5 M NaCl 0.5 M imidazol pH = 7.8

2.11. Recombinant protein refolding

The selected chromatographic fractions were inserted into dialysis tubing VISKING®(Serva) and put into dialysis buffers *(Table 7)* cooled to 4 °C where the concentration of urea decreased from 8 to 0 M. The dialysis buffers were changed in 12 hours intervals. The concentration of the refolded recombinant protein was determined by Bradford assay.

Table 7: Composition of dialysis buffers

Buffer 1	4 M urea 50 mM Tris/HCl pH 7.8 0.5 M NaCl 20 % glycerol 2 mM mercaptoethanol
Buffer 2	2 M urea 50 mM Tris/HCl pH 7.8 0.5 M NaCl 20 % glycerol 2 mM mercaptoethanol
Buffer 3	1 M urea 50 mM Tris/HCl pH 7.8 0.5 M NaCl 10 % glycerol
Buffer 4	50 mM Tris/HCl pH 7.8 150 mM NaCl 10 % glycerol

2.12. Immunization of a rabbit and production of antibodies

An aliquot of purified recombinant protein (100 µg/0.5 mL) was mixed with incomplete adjuvant (0.5 mL) and injected into a rabbit. The immunization was repeated three times in 10 days intervals. After 6 weeks polyclonal serum from the rabbit was obtained [23].

2.13. Obtaining of immunoglobulin fraction

The serum was mixed with two portions of Na-acetate buffer (pH = 4.0) and precipitated by using caprylic acid (25 µL of caprylic acid per 1 mL of the mixture). After the precipitation it was centrifuged (4 °C, 5 000 rpm, 10 min) and filtrated. Obtained supernatant was inserted into dialysis tubing and dialysed in 5 mM Na_2HPO_4 solution in a cold room over night. The protein concentration was determined by Bradford assay and measured at λ = 595 nm. The quality of immune sera, referred to as rabbit x IrAM4, was tested and checked by Western blot analysis.

2.14. SDS-PAGE and Western Blotting

The tissues dissected from six adult females of *Ixodes ricinus* were washed in PBS buffer and homogenized in 1 x DTT solution (gut in 500 µL of DTT, salivary glands in 150 µL of DTT and ovaries in 150 µL of DTT). 10 µL of tissue homogenate, reducing and non-reducing hemolymph or expressed recombinant protein (concentration 1:50) were separated by using SDS-PAGE. Separated proteins were transferred in blotting buffer *(Table 8)* on a PVDF membrane.

Table 8: SDS-PAGE buffer and buffer for Western blotting

Electrophoretic buffer	25 mM Tris 192 mM glycine 0.1 % SDS
5x concentrated sample buffer non-reducing	0.75 M Tris/HCl pH 6.8 5 % SDS 50 % glycerol Traces of bromophenol blue
5x concentrated sample buffer reducing	5x concentrated sample buffer 2.5 % dithiothreitol (DTT)
Staining solution	0.05 % CBB R-250 methanol : acetic acid : water (50 : 10 : 40)
Destaining solution	methanol : acetic acid : water (25 : 10 : 65)
Blotting Buffer	20 % methanol 25 mM Tris-Base 192 mM glycine 0.04 % SDS

2.15. Immunodetection of the recombinant protein

After the electroblotting the membrane was placed into Blocking buffer *(Table 9)*. Membrane and immunoglobulin serum (1:50) with PBS Tween were sealed into foil for 2 hours and then washed with PBS Tween. Secondary antibody conjugated with peroxidase was added for 1 hour. Finally, it was washed with PBS Tween (50 mL). The result was visualized by the developing solution.

Table 9: Solutions used for the immunodetection

Blocking buffer	5 % dry milk solution in PBS
PBS Tween solution (0.05 %)	8 g/L NaCl 0.2 g/L $KH_2PO_4 \cdot 12\ H_2O$ 0.2 g/L KCl 0.05 % Tween 20
Secondary antibody	SwAR/Px (1:1000) (Sevapharma)
Developing solution	0.1 M Tris-HCl pH 7.5 0.6 % 3,3‘-diaminobenzidine 100 µL/100 mL H_2O_2

3. RESULTS AND DISCUSSION

3.1. Amplification of DNA

Total RNA isolated from salivary glands of *Ixodes ricinus* was transcribed into cDNA using anchorched-oligo $(dT)_{18}$ primers and subsequently used as a template for DNA amplification. The amplification was performed by the use of primers derived from known sequence of IsAM4 of *Ixodes scapularis (Table 10)*. Sequencing of the PCR product revealed a high sequence similarity to α_2-macroglobulin from those *Ixodes scapularis* (IsAM4). The nucleotide identity between IrAM4 and IsAM4 was specified to be 95.4 %. Comparison of partial sequences of IrAM4 with IsAM4 is shown in *Figure 2*.

Table 10: The oligonucleotides used in this work and comparison of I.scapularis *genome and* I. ricinus *cDNA sequences*

Amplicon	Forward primer	Reverse primer	Expected size (*I. scapularis* - genome) [bp]	Real size (*I. ricinus* - cDNA) [bp]	Nucleotide identity [%]
IrAM4	5'- GGT CTC TGG ATC TTC AAG GG -3'	5'- GGA CGT ATG CTG TCA GTG G -3'	503	503	95.4

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IsAM4-part     1 GGTCTCTGGATCTTCAAGGGGTGTTTTTACGCTTTCAGGCAACGTCGGGAGTCCCGTGTCAGTGGACAGGCTGGACAAGC
IrAM4-part     1 GGTCTCTGGATCTTCAAGGGGTGTATTTACGCTCTCAGGCAACGTCGGGAGTCCCGTGTCAGTAGACAGGCTGGACAAGC

IsAM4-part    81 TGGTTCGTCTGCCAACTGGCTGCGGGGAGCAGAACCTCGCTTTGCTGGCACCCAACGTCTTTGTGCTCGACTACCTCAAC
IrAM4-part    81 TGGTTCGTCCGCCGACTGGCTGCGGGGAGCAGAACCTCGCTCTGCTGGCACCCAACGTCTTTGTGCTCGACTACCTCAAC

IsAM4-part   161 TCTTCCGGAGAGCGTGGGCATCCCCTGGAGTCAAAGCTGAAAGAGAACATCGCTAAAGGTTACCAGAGGCAACTGAACTA
IrAM4-part   161 TCTTCCGGGGAGCGCGGGCATCCCCTGGAGTTAAAGCTGAAAGAGAACATCGCTAAAGGTTACCAGAGGCAACTGAACTA

IsAM4-part   241 CCGGCACGCCGAAGGCGGCTACAGCGCGTTCGGCTCGCAAGACCCGGAGCCGAGCCTGTGGCTGACTGCATTTGCTGTGC
IrAM4-part   241 CCGGCACGCTGAGGGCGGCTACAGCGCATTTGGCTCACAGGACCCGGAGCCAAGCCTGTGGCTTACTGCGTTTGCTGTGC

IsAM4-part   321 GAACGTTTGGGAGGAGCCGTCGCTTTATGCCTATCGACGAGGCCGAACTCAGCGGCAGCATTCGGTGGATCCTGTCGAAC
IrAM4-part   321 GGACGTTTGGGAGGAGCCGTCGCTTTATGCCCATCGACGAAGCCGAACTCAACGGCAGCATTCGGTGGATCCTGTCGAAC

IsAM4-part   401 CAGTACGACAACGGCTGCTTCCGTCCGTCGGCAGAGTGCTCAACTCAAGATTAAAGGGAGGATTGGAAGGTCATTCCCT
IrAM4-part   401 CAGTACGACAACGGCTGCTTTCCGTCCGTCGGCAGAGTGCTCAACTCAAGATTAAAGGGAGGATTGGAAGGTCATTCCCT

IsAM4-part   481 GGCTCCACTGACAGCATACGTCC
IrAM4-part   481 GGCTCCACTGACAGCATACGTCC
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Figure 2: Comparison of partial sequences of IrAM4 with IsAM4. α_2-Ms were aligned by Megalign program (DNASTAR) and boxshaded.

3.2. Transcriptional profiling

Total RNA isolated from gut, salivary glands and ovaries from partially engorged females of *Ixodes ricinus* was transcribed into cDNA. RT-PCR method was performed for the transcriptional profile detection. The primer set used for RT-PCR was derived from the IsAM4 sequence of the *Ixodes scapularis* genome. Reactions using ferritin as the loading control were detected in all analysed tissues *(Figure 3B)*. RT-PCR showed that IrAM4 is expressed in salivary glands and tick ovaries but it is not present in the tick gut *(Figure 3A)*. A study by Burešová *et al*. [13] showed that IrAM4 is expressed mainly in hemocytes. However, this could not have been investigated in this work due to limited availability of tick hemocytes.

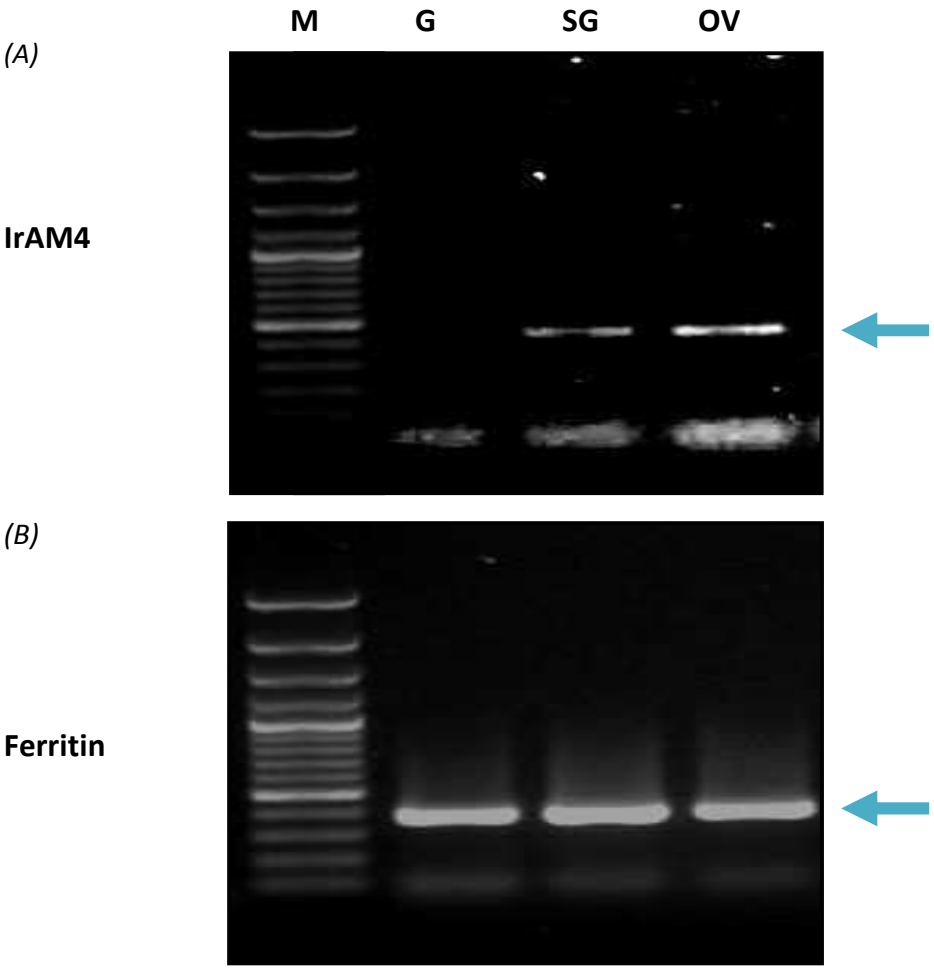

Figure 3: Expression of IrAM4 in various tissues dissected from partially engorged I. ricinus *females*
(A) Expression of IrAM4 in gut, salivary glands and ovaries
(B) Using of ferritin as a loading control
(M) marker, (G) gut, (SG) salivary glands, (OV) ovaries.

3.3. Preparation of recombinant IrAM4 fragment

3.3.1. Recombinant protein expression

The recombinant IrAM4 fragment was expressed in the bacterial sytem of *E. coli*. N-terminal (6x His) fusion protein was prepared using pET100/D-TOPO® expression vector and specific sense and antisense primers *(Table 3)*. Obtained PCR products were isolated from agarose gel *(Figure 4A),* ligated into the expression vector and the resulting expression constructs were transformed into TOP10 Chemically Competent *E. coli* (Invitrogen) cells. Four positive clones were isolated *(Figure 4B)* and the plasmids containing the insert were sequenced to verify the correct open reading frame. The target clone was transformed into One Shot® BL21 Star™ (DE3) Chemically Competent *E. coli* (Invitrogen). The nucleotide and the deduced amino acid sequence of recombinant IrAM4 is shown in *Figure 5*. The theoretical mass of the IrAM4 fragment including the vector part was calculated to be 24 158 Da.

(A) *(B)*

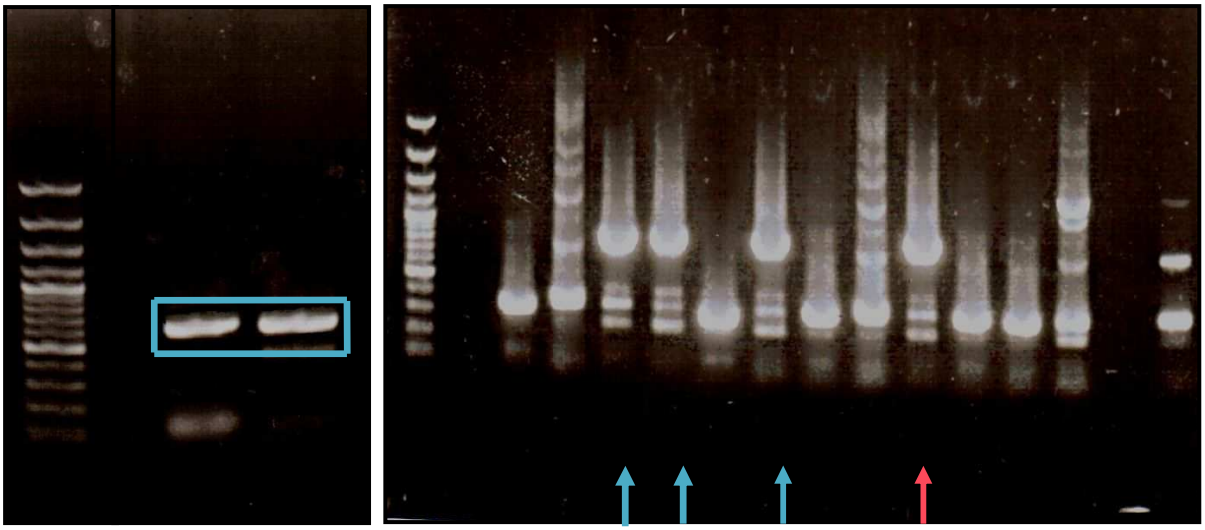

Figure 4: Preparation of recombinant protein expression
(A) Gel electrophoresis and isolation of the PCR product from the agarose gel
(B) Selection of plasmids destined for the sequencing
The red arrow shows the selected clone which was sequenced and used for the further work.

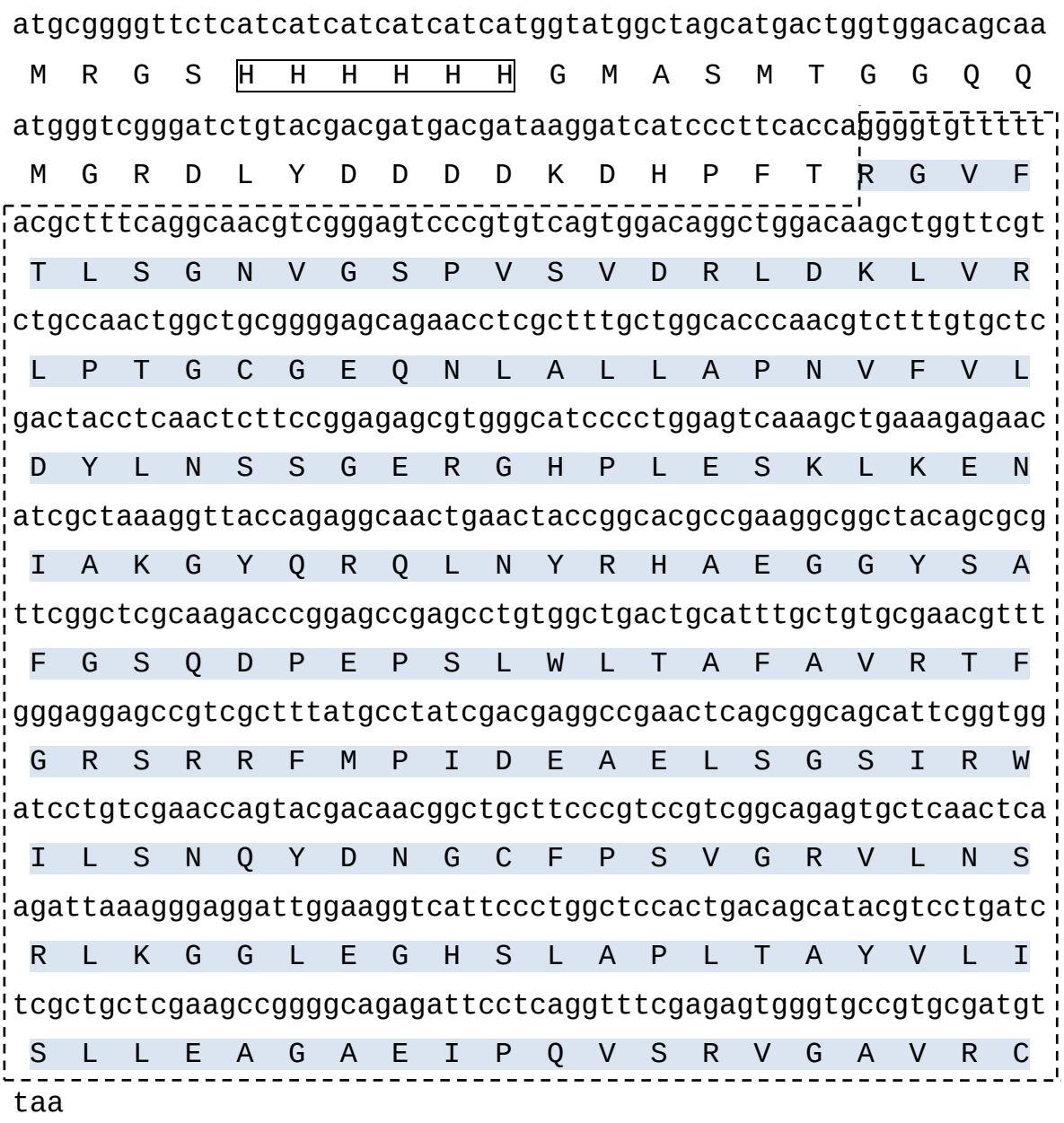

Figure 5: Nucleotide sequence and deduced amino acid sequence of Ixodes ricinus *recombinant IrAM4 fragment.*

3.3.2. Pilot expression and optimization of conditions

Small scale pilot experiments were used to optimize expression levels. Pilot expression was carried out after the transformation of construct into One Shot® BL21 Star™ (DE3) Chemically Competent *E. coli* (Invitrogen) cells by inducing the plasmid with IPTG solution. The cells were then cultivated for six hours at 37 °C. A half milliliter of the bacterial culture was taken away every 1.5 hours and the expression was evaluated by SDS-PAGE on a gradient polyacrylamide gel. The results revealed that expression was already evident after one hour after adding IPTG and the yield of the produced protein increased with time *(Figure 6)*.

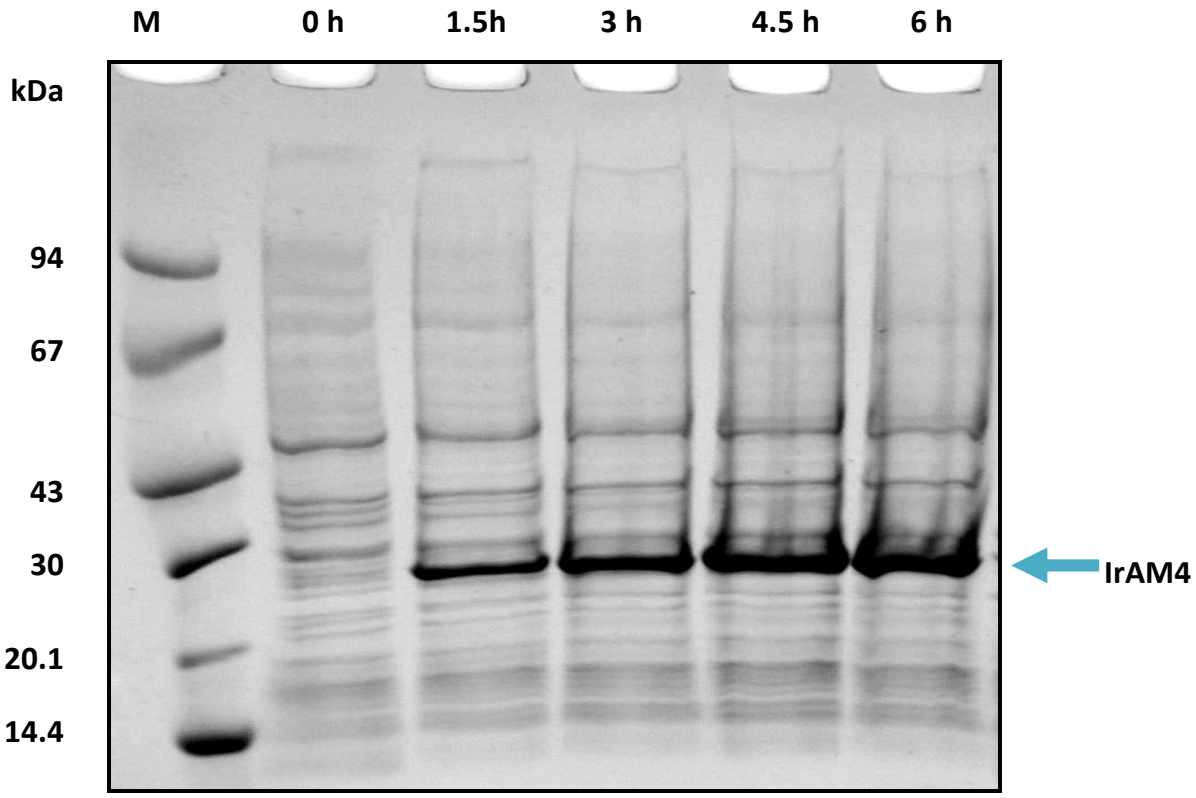

Figure 6: Pilot expression of IrAM4 fusion protein on a gradient polyacrylamide gel (4 - 17.5 %) (M) marker; (0 h) control; (1.5 h, 3 h, 4.5 h and 6 h) time from the beginning of the expression

3.3.3. Isolation of inclusion bodies

The expression of recombinant protein in the system of *E. coli* and subsequent disruption of cells by using detergents and sonification often results in isolation of inclusion bodies containing insoluble aggregates of the expressed protein. The presence of inclusion body impurities could affect the refolding yield of a recombinant protein and thus the inclusion bodies need to be solubilized and purified before refolding. The inclusion bodies were washed and dissolved in guanidine hydrochloride to obtain the soluble form of a recombinant protein. The isolation of the inclusion bodies was checked by SDS-PAGE *(Figure 7)*. After the inclusion bodies had been solubilized the His-tagged recombinant protein was purified by chelate chromatography column.

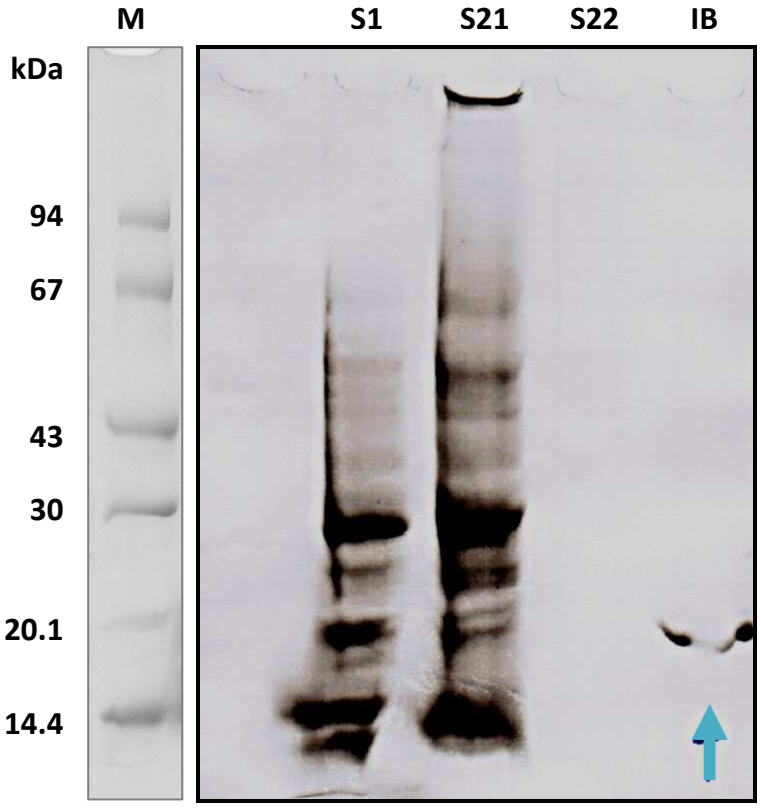

Figure 7: Visualization of the isolated fractions by SDS-PAGE
(M) marker, (S1) cytoplasmic proteins, (S21) and (S22) membrane proteins, (IB) inclusion bodies, the arrow indicates the recombinant protein.

3.3.4. Purification of the recombinant protein

The Histidine-tagged fusion protein was purified from the isolated inclusion bodies by the Co^{2+} chelating chromatography. Buffers contained 8 M urea to maintain denaturing conditions. All purification steps were monitored by absorbance at λ = 280 nm (*Figure 8*). The fractions were collected according to chromatographic profile. The selected fractions A5, A6, A7 and A8 were then used for the protein refolding.

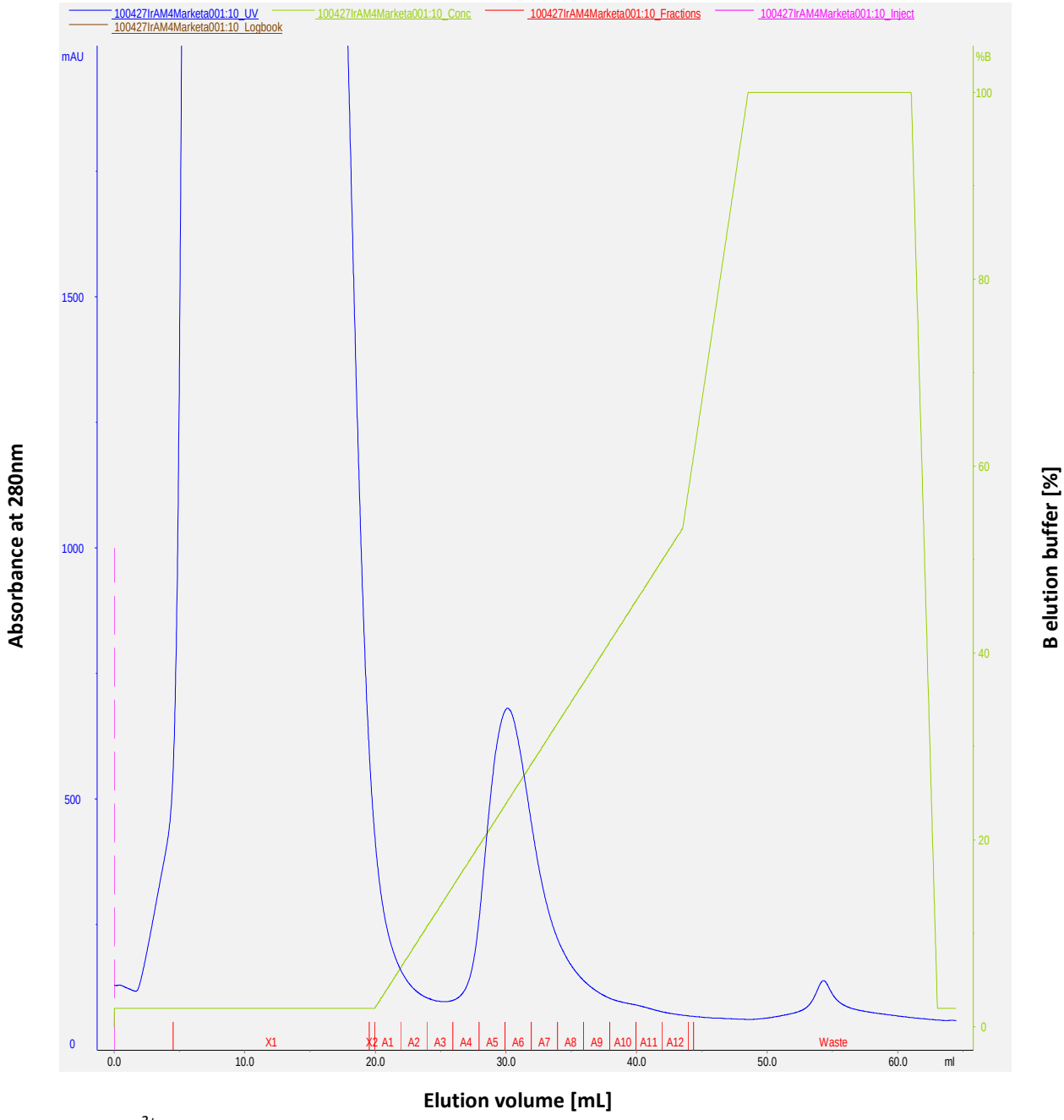

Figure 8: Co^{2+} chelating chromatogram showing the separation of His-tagged protein from contaminating cellular proteins

The separated fractions of the purification process were analysed by SDS-PAGE on a gradient polyacrylamide gel (*Figure 9*). The protein was then refolded in a series of dialysis containing gradually decreased concentrations of urea from 8 to 0 M. At the end the purified recombinant protein IrAM4 was dialysed in Tris/HCl solution with glycerol. A rabbit was immunised with the 4 injections of 100 µg of purified recombinant protein using a standard immunisation protocol [23].

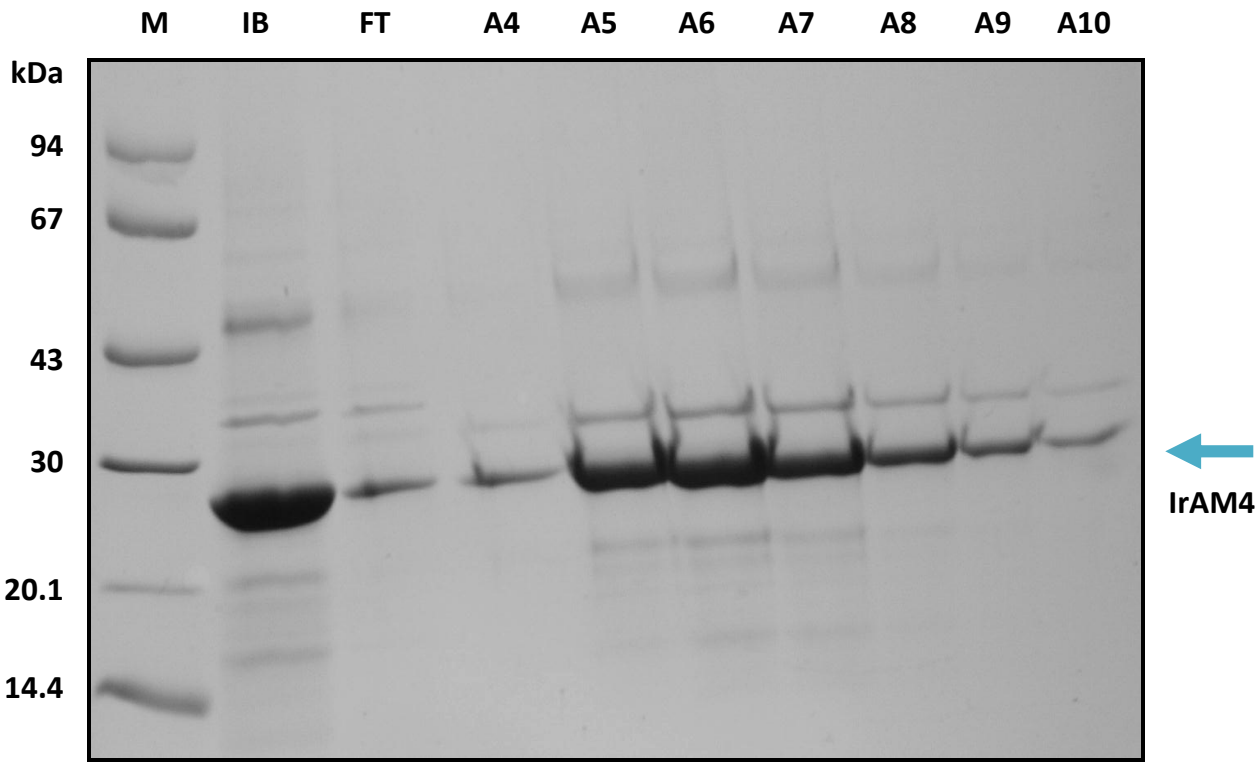

Figure 9. Purification of the recombinant protein - SDS-PAGE on a gradient polyacrylamide gel (4 - 17.5 %) (M) marker, (IB) inclusion bodies, (FT) flow through, A4, A5, A6, A7, A8, A9, A10 are chromatographic fractions. Fractions A5, A6, A7 and A8 were pooled and used for the protein refolding.

After the six weeks the immune serum from the rabbit was obtained. The immunoglobulin fraction was prepared from the immune serum by precipitation with caprylic acid. The quality of Ra x IrAM4 antibodies was tested *(Figure 10)* and the final concentration was chosen according to obtained immunoblot.

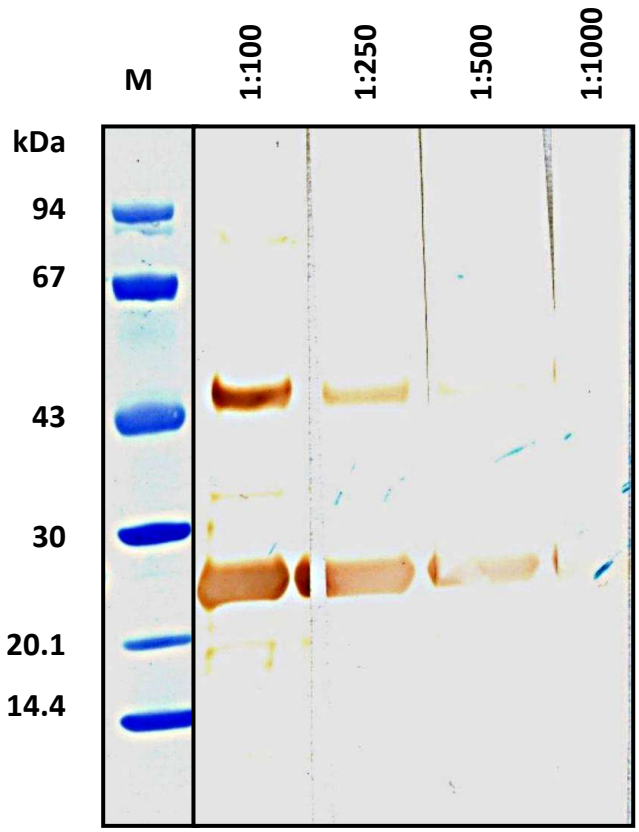

Figure 10: The tested quality of immune sera (Ra x IrAM4) by immunoblotting.
(M) marker, (1:100, 1:250, 1:500 and 1:1000) are the concentrations of the antibodies.

3.4. Detection of authentic IrAM4 in tick tissues

Proteins of homogenized tissues and hemolymph from six partially engorged females of *Ixodes ricinus* were separated using SDS-PAGE *(Figure 11A).* Separated proteins were transfered on a PVDF membrane. Immunodetection was performed with rabbit anti-IrAM4 immunoglobulin fraction dilluted to 1:50. The immunoblot was developed using swine anti-rabbit peroxidase conjugate and diaminobenzidine as a substrate *(Figure 11B)*.

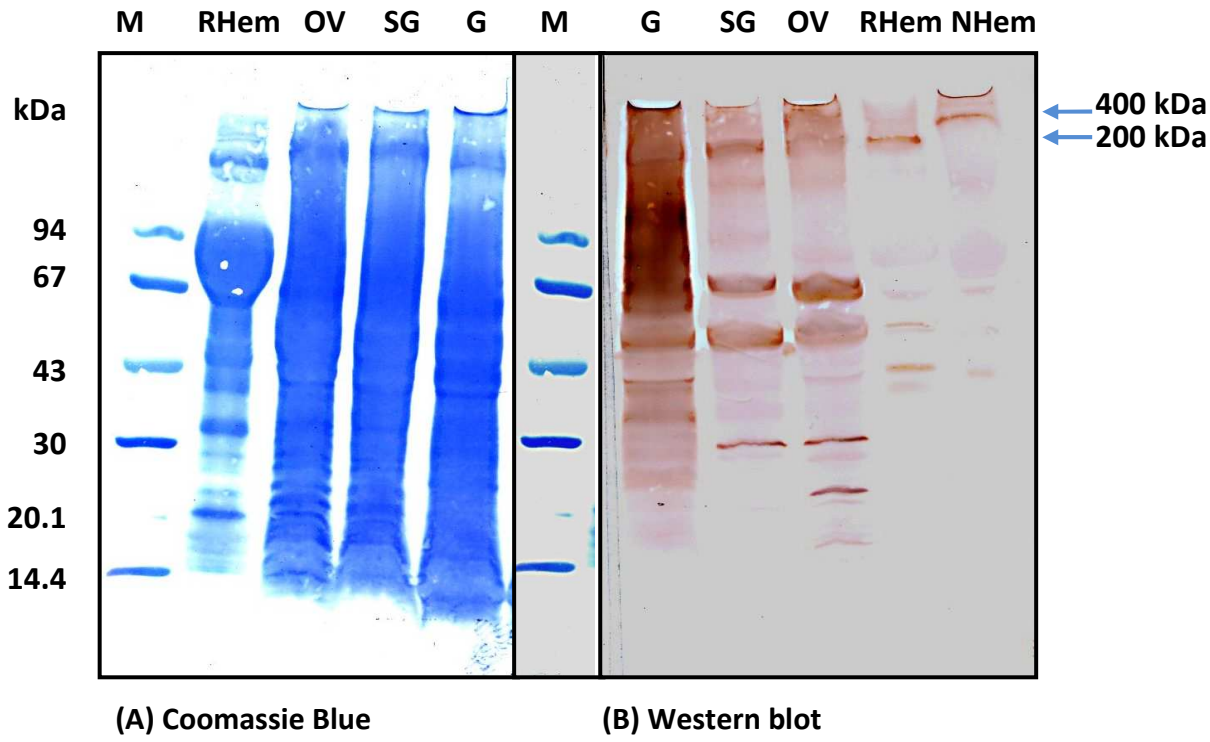

Figure 11: Detection of IrAM4 in tick tissues and hemolymph by Western blotting
(A) Detection of IrAM4 in tick tissues by SDS-PAGE followed by staining with Coomassie Blue
(B) Detection of IrAM4 in tick tissues by immunoblotting. The immunoblot was detected using Ra x IrAM4 immune serum (1:50). The arrows illustrate the position of IrAM4 bands.
(M) marker; (G) gut; (SG) salivary glands; (OV) ovaries, (RHem) reducing hemolymph; (NHem) non-reducing hemolymph

The results of Western blotting experiments predominantly corresponded to the results of RT-PCR analysis. In the case of Western blot analysis the expression in hemolymph was additionally investigated. We observed that IrAM4 is expressed mainly in hemolymph and we detected it in both, a reducing and a non-reducing (without DTT solution) conditions. The size of the band that appeared in the reducing hemolymph was about 200 kDa but in the non-reducing hemolymph there it was about 400 kDa. We therefore assume that IrAM4 is probably composed of two identical subunits bound by disulfide bridges as it is the case for the majority of invertabrate α_2–macroglobulins [5]. The native electrophoresis would be the proper method for a more accurate determination of IrAM4 structure. In other tissues such as ovaries and salivary glands, the antibodies recognized also other bands of lower molecular mass *(Figure 11B)*, which may represent degradation products of IrAM4 or non specific reactions with other proteins. The unambiguous answer to this issue has to await a successful RNA interference experiment.

4. CONCLUSION

In this study, we characterized an α_2-macroglobulin, IrAM4, from the hard tick *Ixodes ricinus.* Total RNA isolated from the homogenated tissues of *Ixodes ricinus* was transcribed into cDNA and used as a template for PCR amplification. Primers were derived from the IsAM4 sequence of the closely related *Ixodes scapularis* genome. Partial sequence was obtained by sequencing of the PCR product. The sequence alignment of IrAM4 with IsAM4 displayed 95.4 % similarity. RT-PCR profiling of tissues from partially engorged females was applied to determine where our desired protein might be present. The RT-PCR profiling revealed that IrAM4 is expressed in salivary glands and ovaries but was not detected in the tick gut. The recombinant protein of IrAM4 fragment was prepared and its theoretical mass was calculated to be 24 158 Da. The purified recombinant protein was used for raising antibodies in a rabbit. After the immunization of the rabbit, polyclonal antibodies were obtained and by using anti-IrAM4 antibodies we were able to detect IrAM4 by Western blot analysis. Authentic IrAM4 was likely present in hemolymph and it was detected under both reducing and non-reducing conditions. The protein appeared as a band of about 200 kDa in reducing hemolymph. However, the band in non-reducing hemolymph corresponeded to the size of 400 kDa. The native IrAM4 seems to be composed of two disulfide bound subunits. The IrAM4 is probably present also in salivary glands and ovaries but for the further analysis other methods as RNA interference or native electrophoresis would be necessary.

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